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METHODS FOR GENERATING DOUBLED HAPLOID PLANTS

Field of the Invention

This invention relates to methods for generating doubled haploid plants from microspores, and to doubled haploid plants produced by the methods disclosed herein.

Background of the Invention

Although plant breeding programs worldwide have made considerable progress developing new cultivars with improved disease resistances, yields and other, useful traits, breeding as a whole relies on screening numerous plants to identify novel, desirable characteristics. Very large numbers of progeny from crosses often must be grown and evaluated over several years in order to select one or a few plants with a desired combination of traits.

In a typical plant breeding experiment, two parent plants are crossed and the resulting progeny (the F1 generation) are screened and a plant (termed the F1 plant) identified that possesses a desirable combination of phenotypic traits. The F1 plant is then self-fertilized to yield a population of progeny plants (termed F2 plants) that must be individually analyzed to determine which F2 plants possess the desired combination of phenotypic traits originally introduced in the F1 plant. If, as is often the case, the desired phenotypic traits derive from the combined effect of several genes, then the number of F2 progeny plants that must be screened depends on the number of genetic differences between the parents of the F1 plant. Thus, the greater the number of

genetically-controlled differences between parents of the F1 plant, the larger the number of F2 progeny that must be grown and evaluated, and the lower the probability of obtaining progeny with all the desired traits.

For example, if the two parents of the F1 plant differ by 25 gene alleles (not an unusually great number in breeding), more land than exists on the earth would be needed to grow all possible genotype combinations which can occur in the F2 generation derived from the self-fertilized F1 plant (Konzak, C.F., Sunderman, D.W., Polle, E.A. and McCuiston, W.L. In: Elliott, L. (ed.) *STEEP—Conservation Concepts and Accomplishment*, pp. 247-273. 1987.). Further, once an F2 plant has been identified that exhibits the same, desirable, phenotypic trait(s) as the cross parents, the process of self-fertilization and analysis of the resulting progeny must be repeated several times until a homozygous population of plants is obtained which breed true for the desired phenotypic character, i.e., all progeny derived from the true-breeding population exhibit the desired, phenotypic trait (though the progeny may not be true-breeding for unselected traits).

One possible solution to the problem of screening large numbers of progeny is to produce them from the gametic cells as haploid plants, the chromosomes of which can be doubled using colchicine or other means to achieve instantly homozygous, doubled-haploid plants. In particular, doubled haploids can be produced from the microspores which normally give rise to pollen grains.

The life cycle of flowering plants exhibits an alteration of generations between a sporophytic (diploid) phase and a gametophytic (haploid) phase. Meiosis produces the first cells of the haploid generation which are either microspores (male) or megaspores (female). Microspores divide and develop within anthers to become mature male gametophytes (pollen). In normal development, microspores are genetically programmed for terminal differentiation to form mature pollen through two cell divisions. However, under certain conditions, microspores can be induced to initiate sporophytic development which leads to the formation of haploid or doubled haploid "embryoids". These embryoids can give rise to mature plants, that are either haploids or doubled haploids, through subsequent sporophytic development. The process by which plants are produced from microspores is termed pollen-embryogenesis or androgenesis, *i.e.*, from the male gametophyte. Androgenesis is of significant interest for developmental genetic research as well as plant breeding and biotechnology, since it is a means to produce genetically true-breeding, doubled haploid plants.

As shown in Table 1, by producing doubled-haploid (also termed polyhaploid) progeny, the number of possible gene combinations for any number of inherited traits is more manageable.

Table 1. Minimum size of F2 population needed to obtain all possible gene combinations with various numbers of independently assorting gene pairs

	Minimum Population Number Required	
Number of Independently Assorting Gene Pairs	Conventional Breeding System	Doubled haploid System
1	4	2
2	16	4
3	64	8
4	256	16
5	1024	32
10	1,048,576	1024
20	1,099,511,627,776	1,048,576

Thus, marked improvements in the economics of breeding can be achieved via doubled haploid production, since selection and other procedural efficiencies can be markedly improved by using true-breeding (homozygous) progenies. With doubled haploid production systems, homozygosity is achieved in one generation. Thus, the breeder can eliminate the numerous cycles of inbreeding necessary by conventional methods to achieve practical levels of homozygosity. Indeed, true homozygosity for all traits is not even achievable by conventional breeding methods. Consequently, an efficient doubled haploid technology would enable breeders to reduce the time and the cost of cultivar development relative to conventional breeding practices.

Thus, there is a need for a method of efficiently producing doubled haploid plants that is applicable to a wide variety of plant species.

Summary of the Invention

In accordance with the foregoing, in one aspect the present invention provides methods for generating doubled haploid and/or haploid plants from microspores.

The methods of the present invention for producing plants from microspores include the steps of: purifying microspores at a developmental stage amenable to

androgenic induction (preferably from mid uninucleate to early binucleate); subjecting the microspores to nutrient stress (and optionally to temperature stress) to obtain stressed microspores; contacting the microspores with an amount of a sporophytic development inducer effective to induce sporophytic development and chromosome doubling, the contacting step occurring before, during, after, or overlapping with any portion of the nutrient stress step; and culturing the isolated, stressed microspores with at least one live plant ovary and/or with an aliquot of plant ovary conditioned medium. Preferably, microspores are contacted with an effective amount of an auxin and/or a cell spindle inhibiting agent before, during, after, or overlapping with any portion of the nutrient stress step. Optionally, microspores are contacted with an effective amount of a gibberellin and/or cytokinin before, during, after, or overlapping with any portion of the nutrient stress step.

In the practice of the methods of the present invention, plant material is selected that bears reproductive organs containing microspores at a developmental stage that is amenable to androgenic induction. Preferably the selected plant material is tillers or branches bearing spikes or flowers that contain microspores in the mid uninucleate stage of development through the early binucleate of development. Preferably the microspores at a developmental stage amenable to androgenic induction are purified by blending, macerating, or otherwise breaking down, the surrounding tissue (such as a wheat plant spike), filtering the ground material through a screen having a mesh size large enough to permit the passage of the microspores, but small enough to prevent the passage of most of the unwanted material, then applying the filtrate, containing the microspores, to a screen having a mesh size that is small enough to prevent the passage of the microspores but which permits the passage of smaller particles and liquid. Presently preferred media in which to blend, macerate, or otherwise break down tissue, including microspores at a developmental stage amenable to androgenic induction, are 0.3M mannitol and 0.25 M maltose.

The purified microspores are preferably subjected to nutrient stress for a period of at least 0.5 hours, preferably from about 0.5 hours to about 120 hours, more preferably for a period of from about 1 hour to about 96 hours, most preferably for a period of from about 24 hours to about 48 hours. A presently preferred method for subjecting the purified microspores to nutrient stress is to incubate the purified microspores in NPB-A medium having the composition described in Table 9 herein. Preferably, the purified microspores are subjected to temperature stress during all or part of the period during which they are subjected to nutrient stress. Preferably

temperature stress is effected by incubating the purified microspores at a temperature of from about 4°C to about 40°C, more preferably at a temperature of from about 28°C to about 35°C, most preferably at a temperature of about 33°C, for a period of from about 0 minutes to about 96 hours, more preferably for a period of from about half an hour to about 24 hours, most preferably for a period of from about one hour to about six hours. Preferably the stressed microspores are then incubated at 27°C in the dark, preferably for up to forty eight hours, before they are transferred to culture medium for embryo development.

The microspores are also contacted with an effective amount of at least one sporophytic development inducer (as further described herein), such as 2-hydroxynicotinic acid (2-HNA), violuric acid, 2-hydroxyproline or ethrel. Preferably the microspores are contacted with an effective amount of a sporophytic development inducer and an effective amount of an auxin (preferably 2,4-dichlorophenoxyacetic acid) and/or an effective amount of a cell spindle inhibiting agent (such as pronamide, amiprophos methyl, dinitroaniline, phosphoric amide and related analogs). The presently preferred concentration range for auxin is from about 0.1 mg/l to about 25 mg/l, more preferably from about 0.2 mg/l to about 10.0 mg/l, most preferably from about 0.5 mg/l to about 4.0 mg/l. The presently preferred concentration range for sporophytic development inducer is from about 0.001 mg/l to about 1000 mg/l. The presently most preferred concentration range for sporophytic development inducer is from about 1 mg/l to about 500 mg/l. The presently preferred concentration range for cell spindle inhibiting agent is from about 1.0 μM to about 200 μM.

Optionally, the microspores are contacted with an effective amount of a cytokinin, preferably kinetin or BAP, and/or an effective amount of a gibberellin. The preferred concentration range for cytokinin is from about 0.1 mg/l to about 10 mg/l, more preferably from about 0.2 mg/l to about 4.0 mg/l, most preferably from about 0.5 mg/l to about 2.0 mg/l. The presently preferred concentration range for gibberellin is from about 0.01 mg/l to about 20 mg/l, most preferably from about 0.2 mg/l to about 4.0 mg/l. The microspores are contacted with some or all of the foregoing chemical agents (sporophytic development inducer, cell spindle inhibiting agent, auxin, cytokinin and/or gibberellin) before, during, after, or overlapping with any portion of the nutrient stress treatment, preferably during the nutrient stress treatment. The isolated, treated microspores are then cultured in a liquid nutrient suspension medium, such as medium NPB98 or NPB 99, preferably NPB 99, supplemented with at least one live plant ovary or an aliquot of plant ovary conditioned medium, until the microspores develop into

embryoids. Preferably the plant ovaries are obtained from wheat varieties "Chris" or "Pavon 76", but ovaries from a wide range of genotypes, and other cereal plant species, including Igri barley and NPB96001 oats, are effective. The embryoids are transferred to a regeneration medium and incubated therein until the embryoids develop into plants. The resulting plants may be doubled haploids, or they may be haploids which can be converted to doubled haploids by treatment with a chromosome doubling agent such as colchicine.

The methods of the present invention for producing plants from microspores may optionally include the step of genetically transforming the microspores. Microspores (preferably uninucleate microspores) can be genetically transformed at any time during treatment of the microspores in accordance with the methods of the present invention. The presently preferred methods of genetically transforming microspores are biolistic gene transfer utilizing a particle gun or electroporation of plasmolyzed microspores. Thus, in one aspect, the present invention provides genetically transformed plants regenerated from microspores.

In other aspects, the present invention provides methods of initiating microspore embryogenesis including the steps of: purifying microspores at a developmental stage amenable to androgenic induction (preferably the mid uninucleate to early binucleate stages of development); subjecting the microspores to nutrient stress (and optionally to temperature stress) to obtain stressed microspores; and contacting the microspores with an amount of a sporophytic development inducer effective to induce sporophytic development and chromosome doubling, the contacting step occurring before, during, after, or overlapping with any portion of the nutrient stress step. Preferably, microspores are contacted with an effective amount of an auxin and/or a cell spindle inhibiting agent before, during, after, or overlapping with any portion of the nutrient stress step. Optionally, microspores are contacted with an effective amount of a gibberellin and/or cytokinin before, during, after, or overlapping with any portion of the nutrient stress step.

In the practice of the methods of initiating microspore embryogenesis of the present invention, plant material is selected that bears reproductive organs containing microspores at a developmental stage that is amenable to androgenic induction. Preferably the selected plant material is tillers or branches bearing spikes or flowers that contain microspores in the mid uninucleate to early binucleate stage of development. Preferably the microspores at a developmental stage amenable to androgenic induction are purified by blending, macerating, or otherwise breaking

down, the surrounding tissue (such as a wheat plant spike), filtering the ground material through a screen having a mesh size large enough to permit the passage of the microspores, but small enough to prevent the passage of most of the unwanted, material, then applying the filtrate, containing the microspores, to a screen having a mesh size that is small enough to prevent the passage of the microspores but big enough to permit the passage of smaller particles and liquid. Presently preferred media in which to blend, macerate, or otherwise break down tissue including microspores at a developmental stage amenable to androgenic induction are 0.3M mannitol and 0.25 M maltose.

The purified microspores are preferably subjected to nutrient stress for a period of at least 0.5 hours, preferably from about 0.5 hours to about 120 hours, more preferably for a period of from about 1 hour to about 96 hours, most preferably for a period of from about 24 hours to about 48 hours. In general, the higher the temperature the shorter is the temperature stress period. A presently preferred method for subjecting the purified microspores to nutrient stress is to incubate the purified microspores in NPB-A medium having the composition described in Table 9 herein. Preferably, the purified microspores are subjected to temperature stress during all or part of the period during which they are subjected to nutrient stress. Preferably temperature stress is effected by incubating the purified microspores at a temperature of from about 4°C to about 40°C, more preferably at a temperature of from about 28°C to about 35°C, most preferably at a temperature of about 33°C, for a period of from about 0 minutes to about 96 hours, more preferably for a period of from about half an hour to about 24 hours, most preferably for a period of from about one hour to about six hours. Preferably the temperature stressed microspores are then incubated at 27°C in the dark, preferably for up to forty eight hours, before they are transferred to culture medium for embryo development.

The microspores are also contacted with an effective amount of at least one sporophytic development inducer (as further described herein), such as 2-hydroxynicotinic acid (2-HNA), violuric acid, 2-hydroxyproline or ethrel. Preferably the microspores are contacted with an effective amount of a sporophytic development inducer and an effective amount of an auxin (preferably 2,4-dichlorophenoxyacetic acid) and/or an effective amount of a cell spindle inhibiting agent (such as pronamide). The presently preferred concentration range for auxin is from about 0.1 mg/l to about 25 mg/l, more preferably from about 0.2 mg/l to about 10.0 mg/l, most preferably from about 0.5 mg/l to about 4.0 mg/l. The presently preferred concentration range for

sporophytic development inducer is from about 0.001 mg/l to about 1000 mg/l. The presently most preferred concentration range for sporophytic development inducer is from about 1 mg/l to about 500 mg/l. The presently preferred concentration range for cell spindle inhibiting agent is from about 1.0 μM to about 200 μM.

Optionally, the microspores are contacted with an effective amount of a cytokinin, preferably kinetin or BAP, and/or an effective amount of a gibberellin. The preferred concentration range for cytokinin is from about 0.1 mg/l to about 10 mg/l, more preferably from about 0.2 mg/l to about 4.0 mg/l, most preferably from about 0.5 mg/l to about 2.0 mg/l. The presently preferred concentration range for gibberellin is from about 0.01 mg/l to about 20 mg/l, most preferably from about 0.2 mg/l to about 4.0 mg/l. The microspores are contacted with some or all of the foregoing chemical agents (sporophytic development inducer, cell spindle inhibiting agent, auxin, cytokinin and/or gibberellin) before, during, after, or overlapping with any portion of the nutrient stress treatment, preferably during the nutrient stress treatment.

The methods of the present invention for initiating microspore embryogenesis may optionally include the step of genetically transforming the microspores. Microspores (preferably uninucleate microspores) can be genetically transformed at any time during treatment of the microspores in accordance with the present invention. The presently preferred methods of genetically transforming microspores are biolistic gene transfer utilizing a particle gun or electroporation of plasmolyzed microspores. Thus, in one aspect, the present invention provides genetically transformed plants regenerated from microspores.

In another aspect of the present invention, doubled haploid and/or haploid plants are provided that are produced according to the methods of the present invention.

Detailed Description of the Preferred Embodiment

The term doubled haploid (abbreviated as DH) is used herein to refer to plants produced by doubling the chromosome number of a gamete-derived haploid plant which is produced via male gamete sporophytic divisions. The chromosome doubling can occur spontaneously at any stage in the process of converting a microspore to a whole plant, or can be induced, for example, by treatment with a cell spindle inhibitor, or after embryogenesis, for example by treating haploid plantlets with colchicine.

The term "microspore" refers herein to the male gametophyte of a plant, including all stages of development from meiosis through formation of the mature pollen grain.

The term "androgenic induction" means induction of androgenesis, *i.e.*, the process by which pseudo-embryos or embryoids, which can be regenerated into plants, are produced from microspores.

The abbreviation mg/l means milligrams per liter.

The methods of the present invention are applicable to a broad range of plant species, including dicotyledonous plants and monocotyledonous plants. Representative examples of plants which can be treated in accordance with the methods of the present invention include, but are not limited to: wheat, barley, rice, corn, triticale, rye, millet, flax, wheat grasses (for example, *Agropyron*, *Elytrigia*), pasture grasses, rye grass, orchard and brome grasses (e.g., *Lolium*, *Phleum*, *Bromus* spp.), turf grasses (*e.g.*, *Poa pratensis*), forage and pasture legumes (e.g., alfalfa, clovers), grain legumes, soybeans, peas, lentils, peanuts, ornamentals including garden and commercial flower and bulb species, fruit trees and nut trees, various vegetable species (*e.g.*, cucurbits, onions, tomato, carrot, potato and other solanaceous plants, beans, peas, lentils), *Brassica* species such as oil seed rape, as well as intergeneric and interspecific hybrids (*e.g.*, triploids, pentaploids, tetraploids, hexaploids, septaploids). The methods of the present invention can be used, for example, to produce inbred lines for use in hybrid seed production, especially to generate vigorous inbred lines from hybrids of crop plants that are susceptible to inbreeding depression. Additionally, the methods of the present invention can be used, for example, to obtain genetically and phenotypically variable progeny from pollen-producing apomictic species, such as blue grasses (*Poa spp.*) and buffalo grasses (*Buchloe spp.*). The methods of the present invention may permit recovery of viable progeny from semisterile plants, such as garlic, Burbank potato, and various self-incompatible species such as cherries, apples, clovers and broccoli.

Preferably, the methods of the present invention utilize wheat spikes as starting material. The methods of the present invention have been successfully applied to spikes from a range of wheat genotypes including, but not limited to: the spring wheats, Calorwa, Chris, Pavon 76, Penawawa, Spillman, Waldron, WED 202-16-2, Wawawai, Red Bobs, SWSW96005, and winter wheats Claire, Eltan, Platte, Enola, Soisson, BonPain, Madsen and Svilena. The presently preferred wheat genotypes are Chris, WED 202-16-2 and Pavon 76. Spring wheat cv. Pavon 76 is generally considered to be a highly androgenic genotype based upon results from anther culture, although it produces a high proportion of albino plants, and a relatively low frequency of spontaneously doubled haploid plants. The androgenic microspores of both Chris and WED 202-16-2 produce high numbers of green progeny, many of which are

spontaneously DH. The presently most preferred wheat cultivar is Chris. Chris is a public variety and Pavon 76 is available from the USDA Cereals Collection, 1691S 2700W, Aberdeen ID 83210. WED 202-16-2 is a *T. dicoccoides*/Pavon 76 derivative from the Volcani Institute, Bet Degan, Israel.

The methods of the present invention permit the production of doubled haploid plants from wheat varieties and cultivars that have long been considered recalcitrant or non-responsive to anther or microspore culture. For example, Waldron is a spring wheat considered recalcitrant to established anther culture methods. Nonetheless, Waldron is responsive to the methods of the present invention.

In many cases, wheat cultivars that are recalcitrant to other methods, such as Waldron and WPB926, can be induced to begin sporophytic divisions at a high frequency utilizing the methods of the present invention. Microspore cell divisions of some of the less responsive genotypes are arrested prior to the emergence of a multi-nucleate pro-embryoid from a common microspore cell wall. A solution to this problem provided by the present invention is to treat plant material containing microspores with novel culture media NPB 98 or NPB 99, the compositions of which are set forth in Table 9. The recalcitrant genotypes respond especially well to culture in NPB 98 or NPB 99 that is conditioned by the prior growth of plant ovaries at a density of 3 to 4 ovaries per milliliter of medium for 7 to 14 days prior to use in microspore culture. Media so conditioned have been shown to accelerate cell divisions and embryogenic development of cultured micrspores. Typically, mature embryoids can be obtained one week earlier than in cultures that utilize whole ovaries instead of ovary-conditioned medium.

An alternative solution to the problem of arrested microspore cell divisions is to make crosses between the recalcitrant cultivars, such as Waldron or WPB 926, and cultivars that efficiently produce green plants from embryoids, such as Chris and WED 202-16-2. In this approach, the methods of the present invention can be incorporated into a more general plant breeding program in which genotypes that are amenable to culture according to the methods of the present invention are crossed with less amenable genotypes which have other, desirable characteristics. For example, Pavon 76, which produces many embryoids, but relatively few green plants when treated in accordance with the methods of the present invention, can be crossed with WED 202-16-2, Chris, or any other wheat genotype which produces a high frequency of green plants from embryoids when treated in accordance with the methods of the present invention. The resulting doubled haploid progeny can be screened for those genotypes

that produce many embryoids and many green plants. Similarly, crossing Chris, Pavon 76 or WED 202-16-2 with a recalcitrant genotype will result in doubled haploid progeny that are amenable to culture according to the methods of the present invention, and which also possess at least some of the desirable traits of the recalcitrant parent. The strategy of crossing a genotype that is amenable to the production of green, double haploid plants with a more recalcitrant plant species, having some other desirable trait(s), is generally applicable to any plant species.

Wheat plants that are used to provide the microspore starting material (referred to as donor plants) in the practice of the presently preferred embodiment of the present invention may be cultivated in the field, but preferably are cultivated in an artificial environment that is less contaminated with microorganisms, such as a greenhouse. Field-grown wheat plants are often heavily infested with microorganisms that contaminate all stages of the microspore embryogenic process. Disinfectant treatment can be used to ameliorate the contamination problem. For example, the starting plant material used in the methods of the present invention can be treated with a 20% (v/v) solution of commercial hypochlorite or chlorine bleach. Any standard growth regime that is known to one of ordinary skill in the art for growing wheat, preferably in a greenhouse, can be utilized in the practice of the present invention.

In general, developing microspores that have at least completed meiosis are useful in the practice of the present invention. Preferably, microspores enclosed within the anthers in the middle section of a spike should be in the mid uninucleate to early binucleate stage of development, more preferably in the late uninucleate to early binucleate stage of development. Microspores that are to be subjected to genetic transformation should preferably be uninucleate. Morphological features of tillers containing microspores at these stages can easily be established for each plant variety by comparing the morphology of the tiller with the microspore developmental stage as determined by microscopic examination with acetocarmine stain. The stages of microspore development are set forth in Bennett, M.D. et al., *Philosophical Transactions of the Royal Society* (Lond.), B issue, Vol. 266, pages 39-81 (1973), which is incorporated herein by reference. The morphology of a wheat tiller is set forth in the following publications, each of which is incorporated herein by reference: Percival, J., *The Wheat Plant, A Monograph*, E.P. Dutton & Co., New York (1921); Montana State University Bulletin 4387, and University of Idaho Series 118.

Preferably the microspores at a developmental stage amenable to androgenic induction are purified by blending, macerating, or otherwise breaking down, the

surrounding tissue (such as the spikelets of a wheat plant), filtering the ground material through a screen having a mesh size large enough to permit the passage of the microspores, but small enough to prevent the passage of most of the ground material, then applying the filtrate, containing the microspores, to a screen having a mesh size that is small enough to prevent the passage of the microspores but which allows passage of the finer particles and liquid. Presently preferred media in which to blend, macerate, or otherwise break down tissue including microspores at a developmental stage amenable to androgenic induction are 0.3M mannitol and 0.25 M maltose. A presently preferred method for isolating microspores is set forth in Example 1.

Isolated microspores are subjected to stress treatment, such as nutrient and/or temperature stress, treatment with sporophytic development inducers, and optionally to treatment with auxins, cell spindle inhibiting agents, cytokinins and gibberellins as is hereinafter further described. Temperature stress is carried out preferably from between about 4°C to about 40°C. The optimum period of stress treatment varies with the genotype. Also, in general, the higher the temperature used to stress the plant material, the shorter will be the time required to temperature stress the plant material. Alternatively, microwave radiation (having an energy of 10^{-6} eV to 10^{-3} eV) can be used to subject the microspores to temperature stress. The absence or reduction of all nutrients, or selected nutrients, for example the absence or reduction of minerals and/or nitrogen, from the water in which the microspores are immersed provides the nutrient stress when desired. Nutrient stress is one way in which to promote the induction of sporophytic development from microspores and can be used, for example, when dealing with microspores from plant genotypes that are resistant to androgenic induction.

In order to increase the yields of androgenic microspores induced by the stress treatments, the microspores are contacted with an effective amount of a sporophytic development inducer. Most preferably, the microspores are contacted with an effective amount of a sporophytic development inducer and an effective amount of an auxin and/or a cell spindle inhibiting agent. Optionally the microspores are contacted with an effective amount of a cytokinin and/or a gibberellin. The microspores are contacted with the foregoing chemical agents before, during, after, or overlapping with any portion of the nutrient stress treatment, preferably during the nutrient stress step.

Sporophytic development inducers useful in the practice of the present invention induce plant microspores to switch from gametophytic development to sporophytic development. By way of non-limiting example, sporophytic development

inducers useful in the practice of the present invention may cause the development of inviable pollen grains, multicellular or multinucleate pollen grains, arrest starch formation in developing microspores and cause physical deformation of mature pollen grains that develop from microspores treated with a sporophytic development inducer. Some sporophytic development inducers useful in the practice of the present invention are chemical hybridizing agents. Chemical hybridizing agents (abbreviated as CHAs) are chemicals which when applied to plants cause the plants to produce inviable pollen. Other sporophytic development inducers useful in the present invention include, but are not limited to: amiprophos methyl, 2-aminonicotinic acid; 2-chloronicotinic acid; 6-chloronicotinic acid; 2-hydroxynicotinic acid; 6-hydroxynicotinic acid; 3-hydroxypicolinic acid; Benzotriazole; 2,2′-dipyridil; 2,4-pyridine dicarboxylic acid monohydrate; 2-hydroxypyridine; 2,3-dihydroxypyridine; 2,4-dihydroxypyrimidine-5-carboxylic acid; 2,4-dihydroxypyrimidine-5-carboxylic acid hydrate; dinitroaniline, phosphoric amide, 2-hydroxypirimidine hydrate; 2,4,5-trihydroxypyrimidine; 2,4,6-trichloropyrimidine; 2-hydroxy-4-methyl pyrimidine hydrochloride; 4-hydroxypyrazolo-3,4,d-pyrimidine; quinaldic acid; violuric acid monohydrate; thymine; xanthine; salicylic acid; sodium salicylate; salicyl aldehyde; salicyl hydrazide; 3-chlorosalicylic acid; fusaric acid; picolinic acid; butanediene monoxime; di-2-pyridyl ketone; salicin; 2,2′-dipyridil amine; 2,3,5-triiodobenzoic; 2-hydroxy pyridine-N-oxide; 2-hydroxy-3-nitropyridine; benzotriazole carboxylic acid; salicyl aldoxime; glycine; D L-histidine; penicillamine; 4-chlorosalicylic acid; 6-aminonicotinic acid; 2,3,5,6-tetrachloride 4-pyridine carboxylic acid; alpha benzoin oxime; 2,3-butadiene dioxime; isonicotinic hydrazide; cupferron; ethyl xanthic acid; 3-hydroxy benzyl alcohol; salicyl amide; salicyl anhydride; salicyl hydroxamic acid; methyl picolinic acid; 2-chloro pyridine; 2,6-pyridine carboxylic acid; 2,3-pyridine dicarboxylic acid; 2,5-pyridine dicarboxylic acid; Monsanto pyridones sold under the trade names Fenridazon and Genesis; pichloram; ammonium thiocyanate; amiben; diethyl dithiocarbamate; glyphosate; anthranilic acid; thiourea; 2,4-dichlorophenoxyacetic acid; 4-chloro anisole; 2,3-dichloroanisole; 2-(2,4)-dichlorophenoxy propionic acid; 2-(4-chlorophenoxy)-2-methyl propionic acid; 2-(para-chloro phenoxy) isobutyric acid and α,β-dichlorobutyric acid.

The presently preferred sporophytic development inducers are: 2-hydroxynicotinic acid (2-HNA); 2-chloroethyl-phosphonic acid (having the commercial name of Ethrel) available from Sigma Chemical Co., PO Box 14508, St. Louis, Mo. 63178-9916; violuric acid monohydrate, 2-chloronicotinic acid and 2-

hydroxyproline. The presently most preferred sporophytic development inducers are 2-hydroxynicotinic acid (2-HNA) and 2-chloroethyl-phosphonic acid. In the practice of the invention, a sufficient amount of the sporophytic development inducer is employed to effect a measurable induction of sporophytic development. The presently preferred concentration range of sporophytic development inducer is from about 0.001 mg/l to about 1000 mg/l.

While not wishing to be bound to a particular theory explaining the method of action of the sporophytic development inducers useful in the practice of the present invention, certain of the presently preferred, representative sporophytic development inducers have some metal chelation ability. In particular, soem of the foregoing, representative sporophytic development inducers can chelate Cu, Mg, Fe and Zn ions. Copper is essential to pollen fertility (Scharrer, K., and Schaumlaufel, E., *Z. Pflanz. Dung. Bodenk*, **89**:1-17 (1960); see also, Tomasik, P., Ratajewicz, Z., In: Newkome, G.R., and Strekowski, L., (eds.) Chapter 3, Pyridine-metal complexes, pp. 186-409 (1986)).

Preferably, the microspores are also contacted with an amount of an auxin effective to maintain callus development. Representative examples of auxins useful in the practice of the present invention include, but are not limited to: 2,4-dichlorophenoxyacetic acid (2,4-D), as well as related auxins (e.g., indoleacetic acid (IAA), indolebutyric acid (IBA), naphthalene acetic acid (NAA), analogues and/or salts of 2,4-D). The presently preferred concentration range for auxin is from about 0.1 mg/l to about 25 mg/l, more preferably from about 0.2 mg/l to about 10 mg/l, most preferably from about 0.5 mg/l to about 4.0 mg/l. The microspores are also preferably contacted with an amount of a cell spindle doubling agent effective to double the chromosome number in a measurable number of microspores. Cell spindle doubling agents (tubulin inhibitors) are auxin-like herbicides of which the presently most preferred is Pronamide (3,5-Dichloro [N-1,1-Dimethyl-2-propynil] benzamide) sold by Chem Service Inc., PO Box 599, West Chester PA 19381-0599. The presently preferred concentration range for cell spindle inhibiting agents is from about 1.0 μM to about 200 μM.

Optionally microspores treated in accordance with the present invention can be contacted with an amount of a cytokinin effective to improve callus quality, in particular to enhance the ability of callus tissue to grow and to increase the size to which callus tissue develops. Representative examples of cytokinins useful in the practice of the present invention include, but are not limited to: kinetin,

benzaminopurine (BAP) and zeatin. Kinetin is the presently preferred cytokinin. Additionally, water in which peeled *Solanum tuberosum* potatoes have been boiled contains significant amounts of cytokinin(s) which can be utilized in the practice of the present invention. The presently preferred concentration range for kinetin, zeatin and BAP is from about 0.1 mg/l to about 10 mg/l, more preferably from about 0.2 mg/l to about 4.0 mg/l, most preferably from about 0.5 mg/l to about 2.0 mg/l.

Optionally, the microspores can be contacted with an amount of a gibberellin effective to enhance cell expansion. The presently preferred concentration range for gibberellin is from about 0.01 mg/l to about 20 mg/l, more preferably from about 0.2 mg/l to about 4.0 mg/l.

The sporophytic development inducer and auxin interact with the aforedescribed temperature and/or nutrient stress treatments to enhance the induction of androgenic microspores. In addition, the sporophytic development inducer and auxin treatments contribute to the completion of androgenesis leading to the eventual formation of mature embryoids which, upon transfer to semi-solid medium, regenerate into green and/or doubled haploid plants. Obtaining enlarged microspores with a fibrillar cytoplasmic structure is a pre-requisite for embryogenesis, but only those that proceed to proembryoids will eventually develop into mature embryoids, which are then able to regenerate to produce plants. Further, it will be understood that the sporophytic development inducers, cell spindle inhibiting agents, auxins, cytokinins, gibberellins and temperature and nutrient stresses described herein act synergistically to produce embryoids from microspores which can be regenerated into whole plants.

In the practice of the the methods of the present invention for producing plants from microspores, the isolated, treated microspores are cultured in the presence of at least one live plant ovary and/or in the presence of an aliquot of plant ovary conditioned medium. Presently preferred sources of ovaries are wheat cultivars Pavon 76 and Chris. It has been found that the plant ovaries do not have to be from the same variety (genotype) or species as the plant from which the embryoids are generated. Thus, for example, ovaries from Igri winter barley, or NPB96001 oats, will stimulate the development of wheat embryoids.

Typically 3 to 6 live plant ovaries will be added to a 60 mm diameter Petri dish containing approximately 5 ml of embryoid culture medium. The presently preferred embryoid culture medium is NPB99, the composition of which is set forth in Table 9 herein. Preferably the embryoid culture medium osmolarity should be around 300 mOsmo.

Embryoids develop from treated microspores, in the presence of at least one plant ovary and/or in the presence of an aliquot of ovary conditioned medium, usually after 3-4 weeks. The developed embryoids are transferred to a regeneration medium, such as media 190-2 and 190-2(b) set forth in Table 9, and the remaining, less-developed embryoids are further incubated with fresh ovaries, and/or ovary-conditioned medium, until mature embryoids form. The presently most preferred regeneration medium is 190-2(b).

During embryoid development, some microspores may die and release substances that are deleterious to embryogenesis. A population that consists almost entirely of live, androgenic microspores can be obtained by the use of a mesh filter with a pore size of 50 μm to filter out dead microspores. This filtration step is typically done about 7 to 10 days after the beginning of coculture of microspores with one or more plant ovaries (and/or culture of microspores in the presence of an aliquot of ovary conditioned medium). The medium containing the microspores is pipetted into an autoclaved 50 μm mesh filter. The 2 to 16 celled, multi-cellular, dividing microspores (and plant ovaries, if present) are retained on the surface of the filter, because their diameters are larger than 50 μm, while the dead microspores pass through the filter. The dividing microspores in the filter are rinsed 3 times with NPB99 medium, and collected by washing the filter, from the reverse side, twice with NPB99 medium (5 ml per wash). These dividing microspores are re-suspended in NPB99 medium, and plated over several plates to accommodate the large number of developing embryos. Nearly all of these microspores are androgenic and will continue their development into embryoids and/or calli. In the experience of the inventors, using the methods of the present invention, microspores obtained from one wheat spike will yield about 5000 to 8000 embryos. A highly purified, androgenic, microspore population is desirable if the microspores are to be genetically transformed, as described below.

Microspores treated in accordance with the methods of the present invention can optionally be genetically transformed by any art-recognized means, for example to produce plants that express one or more desirable traits. Examples of techniques for introducing a gene, cDNA, or other nucleic acid molecule into microspores include: transformation by means of *Agrobacterium tumifaciens*; electroporation-facilitated DNA uptake in which an electrical pulse transiently permeabilizes cell membranes, permitting the uptake of a variety of biological molecules, including recombinant DNA, by microspores; microinjection of nucleic acid molecules directly into microspores; treatment of microspores with polyethylene glycol; and bombardment of

cells with DNA-laden microprojectiles which are propelled by explosive force or compressed gas to penetrate the microspore and enter the cell nucleus.

An example of a microspore transformation technique that utilizes *Agrobacterium tumifaciens* and is broadly applicable to numerous plant species is disclosed in European Patent Application EP 0 737 748 A1. Isolated microspores are cocultivated with *Agrobacterium* containing a Ti plasmid including a transgene (within the transfer DNA of the Ti plasmid) that is to be transferred and stably integrated into the microspore genome. Cellulytic enzymes (such as cellulase, hemicellulase and pectinase) are added during the cocultivation step and serve to permeabilize the microspore cell wall. The transfer DNA (T DNA) is transferred from the *Agrobacterium* cells to the microspores where it is inserted into the microspore genome thereby generating stably genetically transformed microspores. Thereafter, the treated microspores are washed with a mucolytic enzyme (such as lysozyme). Whole plants can then be regenerated from the genetically transformed microspores in accordance with the present invention. Other workers have reported the use of *Agrobacterium* to successfully transform microspores from Brassica (Pechan P.M., *Plant Cell Rep.* **8:** 387-390 (1989); Swanson E.B. and Erickson L.R., *Theor. Appl. Genet.* **78:** 831-835 (1989)).

An example of electroporation-facilitated permeabilization of microspores is reported in Joersbo et al., *Plant Cell, Tissue and Organ Culture* **23:** 125-129 (1990). Joersbo et al. report the transient electropermeabilization of barley microspores to the dye propidium iodide by delivering rectangular electrical pulses to microspores in a chamber with cylindrical coaxial electrodes at a distance of 1 mm. The electroporation treatment had limited deleterious effect on the microspores which could be cultured to produce green plants. Similarly, Fennell and Hauptmann (*Plant Cell Reports* **11:** 567-570 (1992)) reported the electroporation-mediated delivery of plasmid DNA into maize microspores, and also reported the polyethylene glycol (PEG)-mediated delivery of plasmid DNA into maize microspores.

A presently preferred method for stably genetically transforming microspores is biolistic transformation whereby microspores are bombarded with DNA-laden microprojectiles which are propelled by explosive force or compressed gas to penetrate the microspore. Yao et al. (*Genome* **40(4):** 570-581 (1997)) report the production of transgenic barley plants by direct delivery of plasmid DNA into isolated microspores using high velocity microprojectiles. The plasmid used to transform the microspores contained a *bar* gene, under the control of a maize ubiquitin promoter, that conferred

resistance to the herbicide bialaphos. Thus, genetically transformed microspores or embryoids could be selected based on their resistance to bialaphos present in the culture medium. Similarly, Jahne et al. (*Theor. Appl. Genet.* **89:** 525-533 (1994)) also report the production of transgenic barley plants by direct delivery of plasmid DNA into isolated microspores using high velocity gold microprojectiles. Again, genetically transformed microspores or microspore-derived calli were selected based on their resistance to bialaphos present in the culture medium. Fukuoka et al. (*Plant Cell Reports* **17:** 323-328 (1998)) report the production of transgenic rapeseed plants by direct delivery of plasmid DNA into isolated microspores using high velocity microprojectiles. Transformed embryos derived from the microprojectile bombarded microspores were identified by expression of a firefly luciferase gene. Harwood et al. (*Euphytica* **85:** 113-118 (1995))disclose the use of the PDS1000 He particle delivery system to genetically transform barley microspores. The *gus* reporter gene was used to demonstrate both transient and stable transformation events. Additional examples of microspore transformation techniques are set forth in *In Vitro* Haploid Production in Higher Plants, Chapt. 2, Jain et al. (eds), Kluwer Academic Publishers (1996). The aforementioned publications disclosing microspore transformation techniques are incorporated herein by reference, and minor variations make these technologies applicable to a broad range of plant species.

In each of the foregoing transformation techniques, DNA from a plasmid is genetically engineered such that it contains not only the gene of interest, but also selectable and screenable marker genes. A selectable marker gene is used to select only those microspores that have integrated copies of the plasmid (the construction is such that the gene of interest and the selectable and screenable genes are transferred as a unit). The screenable gene provides another check for the successful culturing of only those microspores or microspore-derived embryoids carrying the gene(s) of interest. A commonly used selectable marker gene is neomycin phosphotransferase II (NPT II). This gene conveys resistance to kanamycin, a compound that can be added directly to the growth media on which the microspores grow. Plant cells are normally susceptible to kanamycin and, as a result, die. The presence of the NPT II gene overcomes the effects of the kanamycin and each cell with this gene remains viable. As noted above, another selectable marker gene is a gene, such as the *bar* gene, which confers resistance to a herbicide. A screenable gene commonly used is the -glucuronidase gene (GUS). The presence of this gene is characterized using a histochemical reaction in which a sample of putatively transformed cells is treated with a GUS assay solution. After an

appropriate incubation, the cells containing the GUS gene turn blue. Preferably, the plasmid will contain both selectable and screenable marker genes.

Plants produced in accordance with the methods of the present invention can be doubled haploids, the chromosome number of which doubled during the androgenesis induction phase of development of whole plants from microspores. Additionally, plants produced in accordance with the methods of the present invention can be haploids, the chromosome number of which can subsequently be doubled by treatment with an agent such as colchicine.

In the experience of the inventors, the methods of the present invention are at least approximately 250-fold more efficient than other methods for producing plants from microspores, efficiency being measured as the percentage of microspores that yield green plants.

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention.

EXAMPLE 1

Generating Doubled Haploid Wheat Plants from Microspores

Growing Wheat Plants One to three plants per pot (20 by 25 cm in diameter) are grown in a greenhouse controlled at 29oC +/- 2oC, at a light regime of 17 hours light and 7 hours dark. Fertilizers (N, P, K) are premixed with soil at the time of sowing the seeds. Further application of fertilizer is achieved by daily watering with water containing liquid forms of nitrogen (N), phosphorus (P) and potassium (K) at a ratio 20:20:20 plus minerals. In general, any standard conditions for growing wheat in a greenhouse are acceptable provided that quality donor plants can be harvested. With winter wheats, it is essential that vernalization be complete for normal plant development and appropriate culture responses to be achieved. It is important to note, however, that many winter wheats may devernalize if the greenhouse temperature is above 21oC for as little as three weeks following an artificial vernalization treatment.

Collecting Tillers Fresh tillers that contain microspores at an appropriate developmental stage are cut at a position two nodes below the top of the tiller, and immediately placed in a clean container with distilled water. All leaves are removed by cutting the leaf blades at their bases. The time between the collection of tillers and their treatment is preferably minimized to reduce the possibility of contamination by microorganisms. Microspores enclosed within the anthers in the central section of a spike should preferably be in the mid- to late uninucleate stage of development.

Morphological features of tillers containing microspores at these stages can easily be established for each genotype via microscopic examination with acetocarmine stain. Fresh tillers so collected are then ready for microspore isolation.

Isolation of Microspores for Culture One to six spikes of a given genotype are blended in a Waring MC-II blender, in 50mls of autoclaved 0.25 M maltose or 0.3 M mannitol. After blending, the slurry is passed through a 100 micron filter to eliminate the larger debris. The blender cup is washed three times with 5mls of 0.25 M maltose or 0.3 M mannitol, and the water washings are also passed through the filter. The filtrate is the passed through a 38 micron (μ) filter, which traps essentially all viable microspores and some small debris and dead microspores. The microspores are then washed three times with 5ml NPB-A each, then washed off the filter into a petri dish (60 x 15 mm) using 10mls of water. The total volume here may range from 5 to 10 ml, depending upon the number of spikes blended. The suspension of microspores is then transferred to Petri dishes (60 x 15 mm) at 4 ml each. In each petri dish, one ml of chemical formula stock solution is added. The chemical stock solutions (*i.e.*, containing one or more of sporophytic development inducer, cell spindle inhibiting agent, auxin, cytokinin and/or gibberellin) are prepared as aqueous solutions and filter sterilized. After mixing the microspore suspension and chemical formulation solution, all dishes with microspore suspension are sealed with parafilm and then placed in a 33°C incubator for four to 24 hours followed by an incubation at 27oC for 0 to 48 hours. Alternatively, the suspension of microspores may be incubated only at 27oC for 24 to 72 hours.

The microspores are recovered by passing the treated suspension through a 50μ filter. The microspores are then washed three times with 5ml of solution NPB 98 (having the composition set forth in Table 9). The microspores are washed off into a Petri dish with two 3ml aliquots of solution NPB 98. The microspore density is determined using a hemocytometer, then adjusted to 10,000 microspores per ml of solution NPB 98. The tubes are incubated at 27°C for embryoid induction. The percent microspore survival can be evaluated by adding fluorescein diacetate to the tubes and counting the microspores using a haemacytometer.

Coculture of isolated microspores with plant ovary. Isolated microspores are cultured in liquid NPB 98-1 medium or liquid NPB-99 medium, preferably liquid NPB-99 medium, as a suspension culture. The compositions of NPB 98-1 medium and NPB-99 medium are set forth in Table 6. An aliquot of 2 ml media per 35 mm x 10 mm Petri dish, or 5 ml media per 60 mm x 15 mm Petri dish, at a density of

approximately 1.5 x 10^4 microspores per ml is effective. Immature ovaries are added to the culture at a density of one per ml of medium immediately preceding the incubation. Ovaries are aseptically picked out from fresh and disinfected spikes. Ovaries of all genotypes are effective for the present invention, but the ovaries from varieties Chris or Pavon 76 are preferred for embryogenesis of all wheat varieties tested. Alternatively, medium NPB 98 or medium NPB 9, conditioned by ovaries at a density of three ovaries per milliliter (ml) of medium for 7 to 14 days is mixed directly with microspores without the addition of any fresh ovaries. All Petri dishes are sealed with parafilm and incubated in the dark at 27°C until embryoids of 1-2 mm diameter develop. The embryoids can then be transferred to regeneration medium.

Embryogenic microspores begin their first cell division after approximately 12 hr in culture. Multi-cellular proembryoids, still enclosed within the microspore wall or exine, are formed in approximately one week. In approximately one more week, the exine wall ruptures and immature embryoids emerge, which grow into mature embryoids within about 10 to 14 days. Obtaining enlarged microspores with a fibrillar cytoplasmic structure is a pre-requisite for embryogenesis, but only those that proceed to proembryoids will eventually develop into mature embryoids, which are then able to regenerate to produce plants. The use of some ovary-conditioned medium appears to accelerate embryoid development and influence the uniformity of embryoid development from induced microspores.

When embryoids reach the size of 1 to 2 mm, they are transferred aseptically to solid 190-2 or 190-2(b) medium, preferably 190-2(b) medium, at a density of 25-30 embryoids in each 100 x 15 mm Petri dish. The Petri dishes with embryoids are placed under continuous fluorescent light at room temperature (22°C) for plantlet development. In approximately two weeks, green plantlets develop and are ready for transfer to soil. Green plants are raised in the greenhouse, much like plants grown from seeds. If plants appear to be haploid, colchicine can be applied to induce chromosome doubling. Seeds produced on any plants are instantly homozygous, and so can immediately be used for rapid evaluation and selection in breeding, for analyses in genetics research, or for selection and evaluation of transformants in biotechnology.

EXAMPLE 2

The Effect of 2HNA on Freshly Isolated Microspores

The following experiment was conducted to determine if the presence of 2HNA increases the number of microspores that survive treatment in accordance with the methods of the present invention. Microspores were isolated and treated as described

in Example 1 except that the final density of microspores during the two day 33°C treatment was 6100 microspores per ml. After transfer to NPB98, the microspores were incubated for one day at 27°C. The results of the experiment are shown in Table 2.

Table 2. The effect of various 2HNA concentrations on microspore survival

Concentration of 2HNA (mg/l)	Number of microspores alive after treatment	Total number of microspores after treatment	% microspores alive after treatment
0	0	21	0
10	4	43	9.3
20	5	30	16.7
30	16	76	21.0
40	3	113	2.7
50	2	72	2.8

The data in Table 2 shows that there was a marked effect of 2HNA on microspore survival, particularly at concentrations of 20 and 30mg/l. These results were confirmed and extended in experiments summarized in Tables 3 and 5-8 herein.

EXAMPLE 3

The effect of anthranilic acid and benzotriazole on microspore survival

The following experiment was conducted to determine if the presence of anthranilic acid or benzotriazole increases the number of microspores that survive treatment in accordance with the methods of the present invention. Microspores were isolated and treated as described in Example 1 except that the density of the microspores in solution NPB-A was 7700 per ml. The microspores were counted in microtitre wells. The results of the experiment are shown in Table 3.

Table 3. The effect of anthranilic acid and benzotriazole on microspore survival

Treatment	Number of living microspores after treatment	Total number of microspores after treatment	% of living microspores after treatment
No chemicals	13	80	16.2
0.18mM 2HNA	47	95	49.5

Treatment	Number of living microspores after treatment	Total number of microspores after treatment	% of living microspores after treatment
0.09mM Anthranilic Acid	28	62	45.2
0.18mM Anthranilic Acid	18	69	26.1
0.09mM Benzotriazole	54	145	37.2
0.18mM Benzotriazole	31	63	49.2

These data demonstrate that anthranilic acid at a concentration of 0.09 mM and benzotriazole at a concentration of 0.18mM are both about as effective as 2HNA in promoting microspore survival.

EXAMPLE 4

The effect of sulfanilamide on microspore survival

The following experiment was conducted to determine if the presence of sulfanilamide increases the number of microspores that survive treatment in accordance with the methods of the present invention. Microspores were isolated and treated as described in Example 1 except that the microspore density in solution NPB-A was 1600/ml. Microtitre plate wells were used to determine cell survival. The results of the experiment are shown in Table 4.

Table 4. The effect of various sulfanilamide concentrations on microspore survival

Treatment	Number of living microspores after treatment	Total number of living microspores after treatment	Percentage of living microspores after treatment
No chemicals	33	62	53.2
0.045mM Sulfanilamide	17	42	40.5
0.09mM Sulfanilamide	24	43	55.8
0.13mM Sulfanilamide	28	40	70.0
0.16mM Sulfanilamide	31	63	49.2

The data in Table 4 demonstrate that sulfanilamide at 0.13mM was effective in increasing cell survival.

EXAMPLE 5

The effect of 2,3 Pyridine Carboxylic Acid (2,3 PCA) and 2,3 Butadione Monoxime (2,3 BM) on microspore survival

The following experiment was conducted to determine if the presence of 2,3 pyridine carboxylic acid (2,3 PCA) and 2,3 butadione monoxime (2,3 BM) increases the number of microspores that survive treatment in accordance with the methods of the present invention. Microspores were isolated and treated as described in Example 1 except that ten spikes of Pavon 76 were blended, using two blenders. The 33°C treatment of the microspores in water was for five hours. The microspore density in NPB-A was 2700 microspores per ml. The percent survival was determined one day after transfer to NPB98, using microtitre plates. The results of the experiment are shown in Table 5.

Table 5. The effect of 2,3 PCA or 2,3 BM on microspore survival

Treatment	Number of living microspores after treatment	Total number of microspores after treatment	% of living microspores after treatment
No chemicals	24	66	36.4
0.18mM 2HNA	40	63	63.5
0.09mM 2,3 PCA	33	54	61.1
0.18mM 2,3 PCA	37	69	53.6
0.09mM 2,3 BM	41	70	58.6
0.18mM 2,3 BM	-	-	-

The 0.18mM 2,3 Butadione Monoxime treatment was contaminated. Both 2,3 pyridine carboxylic acid (2,3 PCA) and 2,3 butadione monoxime strongly increased the percentage of microspores living after treatment.

EXAMPLE 6

The effect of 2,4 Dihydroxy Pyrimidine 5 Carboxylic Acid (2,4 DP) and Violuric Acid Monohydrate (VAM) on microspore survival

The following experiment was conducted to determine if the presence of 2,4 dihydroxy pyrimidine 5 carboxylic acid (2,4 DP) or violuric acid monohydrate (VAM) increases the number of microspores that survive treatment in accordance with the methods of the present invention. Microspores were isolated and treated as described in Example 1 except that eleven spikes were blended, using two blenders. The 33°C treatment in water was for 5.5 hours. The microspore density in NPB-A was 5100 microspores per ml, and there were 2ml per treatment. The viability was determined one day after transfer to NPB98. The results of the experiment are shown in Table 6.

Table 6. The effect of 2,4DP5CA or VAM on microspore survival

Treatment	Number of microspores alive after treatment	Total number of microspores after treatment	% microspores alive after treatment
No chemicals	39	74	52.7
0.18mM 2HNA	79	98	80.6
0.09mM 2,4 DP	14	58	24.1
0.18mM 2,4 DP	21	47	44.5
0.09mM VAM	70	97	72.2
0.18mM VAM	48	83	57.8

The violuric acid monohydrate had a very positive effect on microspore survival at a concentration of 0.09mM. In addition, most of the viable microspores from the 0.09mM VAM treatment appeared to be embryogenic, with centrally located nuclei and fibrillar structure cytoplasms.

EXAMPLE 7

The effect of DL Histidine (DLH) and Benzotriazole –5-Carboxylic Acid (B5C) on microspore survival

The following experiment was conducted to determine if the presence of DL histidine (DLH) or benzoatriazole –5-carboxylic acid (B5C) increases the number of microspores that survive treatment in accordance with the methods of the present invention. Microspores were isolated and treated as described in Example 1 except that twelve spikes were blended, using two blenders. The 33°C treatment in water was for 5.75 hours. The microspore density in solution NPB-A was 4600 microspores per ml, two mls per treatment. The results of the experiment are shown in Table 7.

Table 7. The effect of DLH or B5CA on microspore survival

Treatment	Number of microspores alive after treatment	Total number of microspores after treatment	% microspores alive after treatment
No chemicals	99	192	51.6
0.18mM 2HNA	104	152	68.4
0.09mM DLH	118	184	64.1
0.18mM DLH	126	216	58.3
0.09mM B5C	103	179	57.5
0.18mM B5C	139	197	70.6

These data demonstrate that the DL histidine had a beneficial effect on cell survival, although not very many of the viable microspores had a fibrillar appearance. The benzoatriazole-5-carboxylic acid treatments, however, increased both the percent of cell survival and the number of microspores with fibrillar cytoplasm. Most viable cells from these treatments had fibrillar cytoplasm.

EXAMPLE 8

The effect of 2,2 Dipyridyl (2,2 D) and 2,4 Dihydroxy Pyrimidine-5-Carboxylic Acid on microspore survival

The following experiment was conducted to determine if the presence of 2,2 dipyridyl (2,2 D) or 2,4 dihydroxy pyrimidine-5-carboxylic acid increases the number of microspores that survive treatment in accordance with the methods of the present invention. Microspores were isolated and treated as described in Example 1 except that eleven spikes were blended, using two blenders. The 33°C treatment in water was for 5 hours. The density in solution NPB-A was 1600 per ml, two mls per treatment. The viability was determined one day after the microspores were transferred to NPB98. The results of the experiment are shown in Table 8.

Table 8. The effect of 2,2 D or 2,4DP5CA on microspore survival

Treatment	Number of microspores alive after treatment	Total number of microspores after treatment	% microspores alive after treatment
No chemicals	39	70	55.7
0.18mM 2HNA	33	43	76.7
0.09mM 2,2 D	25	39	64.1
0.18mM 2,2 D	39	52	75.0
0.09mM 2,4 DP	30	56	53.6
0.18mM 2,4 DP	41	65	63.1

The 2,2 Dipyridyl had a very beneficial effect on the cell survival, particularly at a concentration of 0.18mM.

EXAMPLE 9

Culture Media

The media set forth in Table 9 are useful in the practice of the present invention. In particular, media NPB98 and NPB-99 are novel media of the present invention and are preferably used to coculture treated microspores with plant ovaries. Medium 190-2(b) is preferably used to culture embryoids during their development

into green plants. Other media, except for MB-97, described in Table 9 are those commonly reported in the literature, but which have been found to be comparatively ineffective for production of embryoids. MB-97 is a novel medium of the present invention and is nearly as effective as NPB98 when used to coculture treated microspores with plant ovaries. The values for the amount of each medium component are milligrams per liter.

Table 9

Chemical (mg/L)	NPB98	MB97	A2	LM94+	MS	CHB-2	MMS-2	NPB-A	190-2	NPB-99	90-2(b
NH_4NO_3				300	1650		300				
$(NH_4)2SO_3$	232	232	231.5			232			200	232	200
KNO_3	1415	1415	1415	1400	1900	1415	1400		1000	1415	1000
$CaCl_2$-$2H_2O$	83	83	83	150	332.2	83	440	148		83	
$Ca(NO_3)2$-$4H_2O$									100		100
KH_2PO_4	200	200	200	400	170	200	170	136	300	200	300
$MgSO_4$-$7H_2O$	93	93	92.5	150	180.7	93	370	246	200	93	200
KCl								1492	40		40
Na_2EDTA	37.3	37.3		37.3	37.3	37.3	37.3		37.3	37.3	37.3
$FeSO_4$-$7H_2O$	27.8	27.8		27.8	27.8	27.8	27.8		27.8	27.8	27.8
Fe-NaEDTA			32					56			
H_3BO_3	5	5	1.5	6	6.2	5	6.2	3	3	5	3
CoCl-$6H_2O$	0.0125	0.0125	0.0125	0.025	0.025	0.0125	0.025			0.0125	
$CuSO_4$-$5H_2O$	0.0125	0.0125	0.0125	0.025	0.025	0.0125	0.025			0.0125	
KI	0.4	0.4	0.375	0.8	0.83	0.4	0.83	0.5	0.5	0.4	0.5
$MnSO_4$-H_2O	5	5	5	11	16.9	5	16.9				
$MnSO_4$-$4H_2O$	5	5						8	8	5	8

Chemical (mg/L)	NPB98	MB97	A2	LM94+	MS	CHB-2	MMS-2	NPB-A	190-2	NPB-99	90-2(b
Na_2MoO_4-$2H_2O$	0.0125	0.0125	0.0125		0.25	0.0125	0.25			0.0125	
$ZnSO_4$-$7H_2O$	5	5	1	9	8.6	5	8.6	0.3	3	5	3
Myo inositol	50	50	50			300	100		100	50	100
Glycine				1		1			2		2
Nicotinic Acid	0.5	0.5	0.5	0.5		0.25	0.5		0.5	0.5	0.5
Pyridoxine-HCL	0.5	0.5	0.5	0.5		0.5	0.5		0.5	0.5	0.5
Thiamine-HCL	5	5	5	1		2.5	0.4			5	
MES			200								
Sucrose									30,000		30,000
Maltose	90,000	90,000	90,000	94,000		90,000	90,000	90,000		90,000	
Ascorbic Acid						0.5					
Biotin				1		0.25					
Casein Hydrolysate				100							
Calcium Pantothenate						0.25					
Glutamine	500	500	500	500		1	146			500	
Kinetin						0.5	0.5	0.5		0.2	

Chemical (mg/L)	NPB98	MB97	A2	LM94+	MS	CHB-2	MMS-2	NPB-A	190-2	NPB-99	90-2(b
2,4-D		1		2		1				0.2	
PAA	1						4			1	
NAA				1							
Gelrite									3,000		3,000

EXAMPLE 10

Use of Plant Ovary Conditioned Medium Instead of Live Plant Ovaries in the Practice of the Present Invention

In the practice of the present invention stressed microspores are cocultured with either ovary-conditioned medium or at least one live plant ovary (although it is within the scope of the present invention to coculture stressed microspores with both ovary-conditioned medium and at least one live plant ovary).

In order to test if media conditioned with ovaries will support the embryogenic development of microspores to completion, ovaries were excised and placed into Petri dishes supplemented with NPB-98 medium. The density of ovaries in the Petri dishes was initially set at ten ovaries per millilitre of medium. After being sealed with parafilm, all Petri dishes were incubated at 27°C for a period of 2 through 45 days, during which the conditioned medium was removed from various dishes at various time points. The removed medium was then mixed with NPB-98 (in a 1:3 ratio) and microspores were isolated. The microspore cultures were maintained at 27°C and examined with an inverted microscope periodically to monitor cell divisions and embryogenic development. Data on the numbers of initial embryogenic microspores, percentage of dividing cells, percentage of proembryoids and of embryoids were collected. The rate of embryoid development was also measured at various time points during culture.

The results of these experiments are shown in Table 10. One dose (1:3 dilution in NPB-98) of ovary conditioned medium was able to support microspore division and carry embryogenesis to completion. Media conditioned with ovaries for 4-21 days were able to sustain microspore embryogenesis. Media conditioned with ovaries for 4 weeks or longer was not able to sustain the cell divisions required to form embryoids, although slow and limited cycles of cell divisions were observed even with 40 d old ovary medium.

Table 10. The Effectiveness of Ovary Conditioned Media in Maintaining Embryogenesis of Freshly Isolated Microspores from Wheat Variety Chris

Conditioning Period (d)	40	37	28	21	14	7	Ovary
2 d Observation*	-MFC	-MFC	-MFC	+MFC	++MFC	++MFC	-MFC
Embryogenic Total %**	20	19	19	22	29	50	41
Cell Division %	10	11	9	18	19	31	41
Proembryoids %	0.8	3.8	4.2	9.1	10.8	22.2	33.5
Embry ids %[a]	0	0	2.2	1.9	9.5	21.9	28.7

* The observations on the morphology of microspores were made after 48 hrs of induction. "-MFC" means that no microspores with fibrillar cytoplasm were observed, whereas "+MFC" means that some microspores with fibrillar cytoplasm were observed. "++MFC" means a large number of microspores with fibrillar cytoplasm were observed. Fibrillar cytoplasm in microspores is a reliable early indicator for the embryogenic potential of microspores. ** All frequencies were estimated based upon the total population of microspores in culture. [a] For culture with fresh ovaries, more embryoids were still developing at the time of collecting data, while all embryoids developed from cultures with ovary conditioned medium had reached the size for transfer. These observations indicate that media conditioned with ovaries for an optimum period accelerate the embryogenic development of microspores compared to co-culture with fresh ovaries.

In a separate experiment, ovaries isolated from genotype Pavon 76 were cultured at a density of ten ovaries/ml of NPB-98. After 7, 14, 35 and 40 days in culture, 0.5, 1.0 or 2.0 ml of ovary conditioned medium was added to microspore cultures to a final volume of 3 ml medium. The dilution of aged (35 or 40 d) ovary conditioned media did not show improved microspore embryogenesis, whereas the same dilutions of younger ovary conditioned media led to decreases in frequencies of proembryoids. These results suggest that the functional ingredients released by ovaries are stimulatory rather than inhibitory in nature (Table 11).

Table 11. The Effect of Dilution of Ovary Conditioned Media on Microspore Embryogenesis

Conditioning Period (d)	**40**			**35**			**14**			**7**		
Conditioned Medium (ml)	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0
Embryogenic Cell %	8	10	12	21	20	23	29	21	44	49	46	52
Proembryoid %	0.2	0.8	0.7	2.5	3.8	3.5	13	16	22	17	22	31
Embryoid %**												

** Data on mature embryoids were not available. However, through repeated early experiments, it is known that the aged media conditioned with ovaries were not able to support microspore embryogenesis to completion while the younger media were able to maintain embryogenesis.

In order to determine the optimum conditioning period of media by ovaries for microspore culture, media conditioned with ovaries at the same density for various time periods were compared with respect to their effectiveness in stimulating microspore embryogenesis. The most effective media were those that were conditioned with ovaries for 7-10 days prior to their use in microspore culture (Table 12). In fact, the ovary conditioned media within this range act to accelerate microspore cell divisions that lead to earlier production of mature embryoids. The first batch of embryoids reach the size of transfer for plant regeneration one week earlier than the control population utilizing fresh ovaries in microspore cultures.

Table 12. The Effect of the Length of Medium Conditioning Period on Embryogenesis of Microspores*

Conditioning Period (d)	40	37	35	28	21	14	10	7	4	ovary
Embryogenic %	20	19	17	19	22	34	47	50	49	41
Proembryoid %	0.8	3.8	2.1	4.2	9.1	16	25.8	22.2	26.2	33.5
Embryoid %	0	0	0	2.2	1.9	15.5	23.4	21.9	16.3	28.7

* Microspores were isolated from Pavon 76 while ovaries were obtained from genotype Bob White.

Additionally, a wide range of ovary densities were tested to determine the number of ovaries in the conditioning medium that is most effective for stimulating microspore embryogenesis once the conditioning medium is diluted to a final working concentration in microspore cultures. Microspores were isolated from Pavon 76 as described herein. Five to 30 ovaries per ml of original conditioning medium were all effective, 4 ovaries/ml or below were sub-effective (Table 13). On this basis, the ovary conditioned medium is as effective as the same number of fresh ovaries placed directly in the culture medium at the time cultures are initiated. Twenty ovaries per ml of medium seemed to be most effective, saturating the demand for active ingredients released by cultured ovaries. Ten ovaries per ml worked as well, thirty were no better than twenty. Twenty ovaries per ml of conditioning medium (subsequently diluted three-fold to achieve a working concentration) is effective for all wheat genotypes tested thus far, including Pavon 76, Chris, WED 202; 16-2, Bob White and WPB 926.

Table 13. The Effectiveness of Media Conditioned by Ovaries at Various Densities

Ovaries/ml	Ovary	2	5	10	15	20	25	30
2 d Observation*	-MFC	-MFC	-MFC	+MFC	+MFC	++MFC	++MFC	++MFC
Embryogenic Total %	41.3	3.1	31.8	50.0	41.7	49.5	51.2	47.3
Proembryoid %	33.5	<0.5	13.7	22.2	20.8	24.9	23.8	25.4
Embryoid %	14.7**	0	11.6	21.9	18.2	23.6	23.1	22.7

* Observations were made using an inverted microscope 2 days (d) after the initiation of culture. "-MFC" represents no microspores with fibrillar cytoplasm; "+MFC" indicates the presence of some microspores with fibrillar cytoplasm; "++MFC" represents the presence of a large number of microspores with fibrillar cytoplasm. ** At the time that these data were collected, petri dishes supplemented with fresh ovaries rather than extract had more embryoids growing but these had not yet reached the size for transfer.

In order to determine the effect of ovary genotype on the effectiveness of ovary-conditioned medium in the practice of the present invention, ovaries were excised from various genotypes and were tested for their effectiveness in stimulating microspore embryogenesis. Microspores were isolated from wheat variety Chris. The available data do not show genotype differences. Media conditioned with ovaries from all genotypes, ranging from early uninucleate through mature pollen were all effective for promoting the production of proembryoids from microspores. The density of ovaries and the time they were cultured in the conditioning medium were more critical than the genotype and the developmental stage of ovaries at the time of excision. Ten ovaries per ml of medium conditioned for a period of 7-14 days, and subsequently diluted three fold for microspore culture were the most beneficial for embryoid development.

EXAMPLE 11

Reducing the Number of Albino Plants

Although the methods of the present invention produce high yields of embryoids from the microspores of a wide range of plant species, embryoids from some plant genotypes yield a high percentage of albino plants. Albino plant percentage can be reduced by lower temperature treatment during the temperature stress period, but the total number of embryoids produced is sharply reduced and the total number of green plant production is consequently low.

The level of nutrients available to the microspores during the nutrient stress step of the methods of the present invention was assessed for its effect on the number of

green plants produced. Three spikes of spring wheat line WED 202-16-2 were treated in a flask containing 50 ml of 100 mg/l 2 HNA, 10 mg/l 2,4-D, 2 mg/l BAP, 3 mg/l GA with or without 10% NPB98 induction medium at 33°C for 69 hours. Microspores were released as described herein. Microspores were cultured in 5 ml of NPB98 induction media in 6 cm Petri dishes at a density of 30,000 microspores per ml induction medium. Four fresh ovaries of WED 202-16-2 were added to each of the Petri dishes. There were 2 replications for each treatment. The results are shown in Table 14.

Table 14

Pretreatment media in flask		With 10% NPB98	Without 10% NPB98
# of embryoids	Rep1	500	500
	Rep2	500	500
	Mean	500	500
# of 1st group of embryoids	Rep1	50	50
transferred to 190-2	Rep2	50	50
	Mean	50	50
# of green plants	Rep1	30	21
	Rep2	40	23
	Mean	35	22
Green plant regenerant (%)	Rep1	97	70
	Rep2	95	70
	Mean	96	70
# of albino plants	Rep1	1	9
	1 Rep2	2	10
	Mean	2	10
Albino plant regenerant (%)	Rep1	3	30
	Rep2	5	30
	Mean	4	30

Pretreatment media in flask		With 10% NPB98	Without 10% NPB98
# of 2nd & 3rd group of embryoids* transferred to 190-2	Rep1	65	65
	Rep2	65	65
	Mean	65	65
# of green plants	Rep1	32	23
	Rep2	42	32
	Mean	37	28
Green plant regenerant (%)	Rep1	82	70
	Rep2	84	71
	Mean	83	71
# of albino plants	Rep1	5	10
	Rep2	8	13
	Mean	7	12
Albino plant regenerant (%)	Rep1	18	30
	Rep2	16	29
	Mean	17	30

* When transferring the first group of embryoids, all culture plates were refreshed with 2.5 ml new NPB98 induction media, and the old ovaries were also replaced with fresh ovaries from the genotype Chris at a density of five ovaries per plate.

The results clearly show that the addition of 10% NPB98 induction medium in the pretreatment medium resulted in an increase of total green plant production, and a decrease of albino plant formation.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.